Accepted Manuscript

Use of ^{31}P NMR and FTIR to investigate key milk mineral equilibria and their interactions with micellar casein during heat treatment

Mattia Boiani, Mark Fenelon, Richard J. FitzGerald, Phil M. Kelly

PII: S0958-6946(18)30035-9

DOI: 10.1016/j.idairyj.2018.01.011

Reference: INDA 4271

To appear in: *International Dairy Journal*

Received Date: 15 November 2017

Revised Date: 16 January 2018

Accepted Date: 20 January 2018

Please cite this article as: Boiani, M., Fenelon, M., FitzGerald, R.J., Kelly, P.M., Use of ^{31}P NMR and FTIR to investigate key milk mineral equilibria and their interactions with micellar casein during heat treatment, *International Dairy Journal* (2018), doi: 10.1016/j.idairyj.2018.01.011.

Use of ^{31}P NMR and FTIR to investigate key milk mineral equilibria and their interactions with micellar casein during heat treatment

Mattia Boiani [a,b], Mark Fenelon [a], Richard J. FitzGerald [b], Phil M. Kelly [a*]

[a] *Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland*

[b] *Department of Biological Sciences, University of Limerick, Castletroy, Limerick, Ireland*

*Corresponding author. Tel: +35 387 7735352

E-mail address: phil.kelly@teagasc.ie (P. M. Kelly)

ABSTRACT

The thermal treatment of milk is one of the key processes routinely performed in the dairy industry. Several modifications occur in milk during heating, particularly with respect to its mineral equilibrium. As the temperature increases, the solubility of calcium and phosphate decreases leading to precipitation in the casein micelle as casein phosphate nanocluster. Recently, ^{31}P NMR and Fourier Transform Infrared have been demonstrated to be capable of monitoring changes to its nanocluster. In this study, the effect of temperature on nanocluster during heating of milk to temperatures ranging from 25 °C to 80 °C followed by subsequent cooling were studied. It was also demonstrated that key ionic components of the mineral equilibria behaved differently with temperature, e.g., calcium influence was evident only at lower temperature, while the opposite was the case with phosphate. It was also shown that micellar casein concentration was influential at all temperatures, most notably at lower values.

1. Introduction

The complexity of milk makes its original composition easily susceptible to modification during different industrial processes. One of the more important industrial factors is temperature arising from thermal processing (Dalgleish & Corredig, 2012). The main reasons for heating milk are to (a) kill pathogens (pasteurisation), (b) increase its shelf life (Fox, Uniacke-Lowe, McSweeney, & O'Mahony, 2015), (c) increase the solids content by means of thermal evaporation before spray drying (Le Graet & Brule, 1982; Liu, Dunstan, & Martin, 2012) and (d) influence the heat classification of skim milk powder produced subsequent to the use of various preheat temperatures prior to evaporation.

An extensive range of temperatures is used during processing depending on application, e.g. from modest levels of heating (40–50 °C) during the advanced stages of milk evaporation (Liu et al., 2012) to values exceeding 130–140 °C in the case of ultra high temperature (UHT) processing (Fox et al., 2015). In addition, different heating systems feature different holding times. Previous studies showed how heating milk to certain temperatures influences the interaction of whey protein with casein micelles (Corredig & Dalgleish, 1996) and causes shifts in mineral equilibria (Gaucheron, 2005). Complex formation between whey protein and micellar casein during heat treatment relies extensively on the exposure of a thiol group during whey protein unfolding, denaturation and its resulting interaction with κ-casein (Donato & Guyomarc'h, 2009). However, in this study we focus on changes to the mineral equilibria in the course of heating milk between 25 °C and 80 °C. With increasing temperature, the solubility of calcium (Ca) and inorganic phosphate (P_i) in solution is reduced, leading to precipitation of phosphate and calcium (On-Nom, Grandison, & Lewis, 2010; Pouliot, Boulet, & Paquin, 1989a). At the same time, the pH of milk reduces within this temperature range (On-Nom et al., 2010). This change was found to be completely reversible if heating was conducted at 80 °C (Pouliot, Boulet, & Paquin, 1989b), but once it reaches > 90 °C an increase in soluble phase occurs (Wahlgren, Dejmek, & Drankenberg, 1990; Zhang & Aoki, 1996).

A limitation with studies to date on the influence of temperature on milk minerals is that they have been confined to milk as whole system. However, the mineral equilibria of milk are more complicated than a single equation (Holt, 2004). This study set out to show how key components of the mineral equilibria of milk respond differently when temperature increases. The experimental approach utilised ^{31}P nuclear magnetic resonance (^{31}P NMR) and attenuated total refraction Fourier transform infrared (ATR FTIR), since both techniques are capable of directly probing the casein phosphate nanocluster structure as well as the repartitioning of phosphorus (Boiani, McLoughlin, Auty, FitzGerald, & Kelly, 2017; Boiani, FitzGerald, & Kelly, unpublished). A better understanding of the influence of heat treatment on the different components of milk is intended to provide dairy industry personnel with additional knowledge on how to better control physicochemical and functional properties during the manufacture of milk products.

2. Materials and methods

2.1. Sample preparation

Pasteurised skimmed bovine milk obtained from the local market was used as reference sample during investigation on temperature effects on milk salt equilibria. A further three samples were prepared by increasing, respectively, the concentrations of micellar casein, P_i, and P_i + Ca. The concentrated micellar casein (milk concentrate) sample was obtained by microfiltration (MF) at 25 °C and 1 bar using a NovaSet™-LS membrane (TangenX, Shrewsbury, MA, USA), starting with 1 L of skimmed milk and collecting 0.5 L of permeate. The samples with higher mineral concentrations were obtained by adding 10 mmol of sodium phosphate monobasic to 1 L of skimmed milk or 10 mmol of sodium phosphate and 10 mmol calcium chloride. Samples were left for 1 h to equilibrate at room temperature under gentle magnetic stirring following each mineral addition. After 1 h initially, and every 30 min subsequently, the pH was corrected using 1 M NaOH

to the initial value of skimmed milk (pH = 6.8) until stabilised. Sodium azide as added (0.3 mg mL^{-1}) to all samples to control microbial growth and stored overnight at 4 °C before analyses.

2.2. SDS PAGE analysis

The protein profiles of the samples and their distribution between retentate and serum fractions were assessed using reducing SDS PAGE. Gels of total protein and soluble fractions were performed using diluted samples with the Nu PAGE® SDS reducing buffer and obtained as already described in Boiani et al. (2017) using 12% bis-Tris precast gels (1.0 mm × 10 well; Novex® by Life Technologies™, Carlsbad, CA, USA).

2.3. NMR spectroscopy

^{31}P NMR spectra were collected using a 500 MHz Bruker spectrometer (Bruker UK Ltd, Coventry, UK) using an external phosphate solution as reference. Data acquisition was as previously described in Boiani et al. (2017). Three different spectra were collected for every sample: a first spectrum at 25 °C was collected as soon as the sample was loaded on the spectrometer. The sample was then warmed within the NMR spectrometer to collect the spectrum at higher temperatures. The skimmed milk, milk concentrate and milk plus P_i and Ca samples were investigated at seven different temperatures: 25, 40, 60, 65, 70, 75 and 80 °C. The milk plus P_i was investigated at 25, 35, 40, 45, 60, 65, 80 °C. Immediately following collection of the second spectrum, the NMR spectrometer and its entrapped sample, were cooled to 25 °C whereupon a third spectrum was collected to establish the effect of temperature reversibility. Each spectrum involved a 4 h collection time, thus, exposing the sample to a total time of 12 h in the spectrometer. Lock of the signal and topshim command was executed every time a new spectrum was gained. All spectra were analysed using Top Spin 3.2 software (Bruker UK Ltd). ^{31}P NMR casein phosphate

nanocluster peak areas were identified using P_i as reference and used to investigate the influence of the heating on the concentration of both phosphorus species.

2.4. FTIR spectroscopy

FTIR spectra were generated to investigate the recovery behaviour of samples after 80 °C for 4 h. The FTIR spectrometer used was a 27 Tensor FTIR (Bruker UK Ltd, Coventry, UK) equipped with an ATR BioATRCell II probe (Bruker UK Ltd, Coventry, UK). The spectra were collected as previously described (Boiani et al., unpublished), except that MF permeate was used instead of water as background sample. MF permeate was obtained from the microfiltration of milk. This microfiltration was similar to that used for preparation of the casein concentrate except that in this case the retentate was not recycled to the feed vessel during operation. After discarding an initial 250 mL of permeate, 3 mL of permeate was collected for use as background. All spectra manipulations were conducted using OPUS 5.5 (Bruker Optik GmbH, Ettlingen, Germany) software.

2.5. pH measurement

Sample pH was determined using a Mettler Toledo pH meter (Mettler-Toledo Ltd., Beaumont Leys, Leicester, UK). The pH meter was calibrated with standard pH solutions. pH measurements were taken at 25 °C.

2.6. Statistical analysis

Samples were analysed in duplicate for pH and NMR peaks area. Analysis of variance (ANOVA) was undertaken using Minitab version 17 (Minitab Inc.). The level of significance was

established at $P < 0.05$. Fisher's multiple-comparison test was used for paired comparison of treatment means and the level of significance was determined at $P < 0.05$.

3. Results

3.1. Influence of temperature on milk minerals equilibria

The mineral equilibria of milk are influenced by temperature. As temperature increases, the shift towards the colloidal/solid phase (Pouliot et al., 1989a) may be observed in the spectrum generated by ^{31}P NMR along with changes to the casein phosphate nanocluster (Fig. 1). P_i and the casein phosphate nanocluster are visible at 1 ppm (sharp high intensity signal) and 2–3.5 ppm (multiplet), respectively (Boiani et al., 2017). ^{31}P NMR analysis also shows that increasing the temperature of skimmed milk results in an initial reduction in the P_i peak signal, however at temperatures > 70 °C a shift in the peak to higher ppm was visible (Fig. 2). At the same time, increasing temperature shifts the casein phosphate nanocluster peaks to lower ppm and expands their peak area (Fig. 3). However, this increase in casein phosphate nanocluster area was not linear and two different trends emerged (see Fig. 1 and Table 1). The initial increase in temperature had a minor effect on casein phosphate nanocluster change as reflected by the value (0.0011) of the initial slope of the linear regression; however, once the temperature exceeded 70 °C increased precipitation of P_i to casein phosphate nanocluster raised the linear regression slope to 0.0085. The intercept of the two linear regression lines corresponds to a temperature of 68 °C (Fig. 1; Table 1). By analysing the combined set of ^{31}P NMR and FTIR results, it was possible to explore the influence of temperature on the recovery of the mineral equilibria (Fig. 4A,B). In Fig. 4A, no statistical difference ($P > 0.05$) was observed within the area of casein phosphate nanocluster during recovery at 25 °C; however, a comparison of the FTIR spectra of skimmed milk before and after 80 °C (Fig. 4B) clearly shows a reduction of the casein phosphate nanocluster signal at 1051 cm^{-1}.

3.2. Casein micelle, phosphate and calcium influence

While the effect of heat treatment has been investigated extensively in the case of milk (Gaucheron, 2005), its influence on the behaviour of individual components of the mineral equilibria has yet to be studied in detail. It was, therefore, hypothesised that by increasing the concentration of micellar casein, P_i and Ca, their respective influences would be accentuated at the different experimental temperatures, and hence, shed additional light on their individual roles. Thus, it was decided to focus on three components: micellar casein, P_i and the Ca, all three of which are directly involved in the formation of casein phosphate nanocluster.

3.2.1. Micellar casein

A higher concentration of micellar casein increases casein phosphate nanocluster availability not only in the system but at the micellar core where P_i and Ca most likely aggregate with increasing temperature. By means of MF, it was possible to increase the concentration of micellar casein while maintaining the concentration of all other components equal to that of skimmed milk. As expected, an increase in micellar casein concentration caused an increase in the casein phosphate nanocluster area signal in the ^{31}P NMR spectrum (Fig. 1) such that the slope of the linear regression at low temperature (0.0032) became three times higher than that of skimmed milk, and contrasted with that of the upper temperature range where there was a 2× increase in the value (0.0175) of the slope (Table 1). At the same time, the intercept of the two linear regression lines is at 69 °C, close to that found in skimmed milk (Table 1). When studying the reversibility of the temperature effects (Fig. 4C,D) following cooling to 25 °C, the reduction in casein phosphate nanocluster signal intensity observed after exposure to higher temperature was in agreement with the findings of previous workers (de la Fuente, 1998), while the FTIR spectrum (Fig. 5D) showed a similar reduction to that of skimmed milk (Fig 4B).

198

199 *3.2.2. Phosphate*

200 An increase in the concentration of phosphate has a dual influence, i.e., an effect on the
201 process of casein phosphate nanocluster formation and at the same time a role in calcium chelation
202 (Gaucher, Piot, Baucher, & Gaucheron, 2007). The amount of P_i added during experimentation was
203 set at 10 mM, to double the milk concentration (Gaucheron, 2005) of soluble phosphorus. As
204 expected, phosphate supplementation reduced the amount of casein phosphate nanocluster (Fig. 1)
205 based on the area of P_i as reference. Increasing P_i had a minor effect on the low temperature slope
206 (0.0008), while at high temperature the slope (0.002) was four times less than that of skimmed milk
207 (Table 1). The two regression lines intercepted at 56 °C compared with 69 °C for skimmed milk
208 (Table 1). Following temperature reversal to 25 °C, ^{31}P NMR analyses of the recovered samples
209 showed (Fig. 4E), with exception of the sample heated to 80 °C, that the casein phosphate
210 nanocluster content remained unchanged with reference to that of skimmed milk. However, when
211 the 80 °C heated sample was cooled to 25 °C an increase in the casein phosphate nanocluster signal
212 was detected by ^{31}P NMR, but not by FTIR (Fig. 4F). When the recovery effect was further studied
213 using SDS PAGE (Fig. 5), it was possible to observe an increase in soluble casein protein in the
214 supernatant of milk plus P_i sample after heating at 80 °C (Fig. 5C).

215

216 *3.2.3. Calcium*

217 As in the case of phosphorus, calcium ions have a double effect in milk – formation of
218 casein phosphate nanocluster and contribution to the supramolecular structure of micellar casein
219 and subsequent functionality in dairy product processing, e.g., coagulation (McMahon, Brown,
220 Richardson, & Ernstrom, 1984). For this reason, it was decided not to investigate calcium alone but
221 rather in combination with phosphate to avoid coagulation during analysis. P_i (10 mM) followed by
222 Ca (10 mM) were added to skimmed milk as outlined in the Materials and methods section. When
223 Ca was added to the P_i supplemented milk and subjected to the NMR heating, the linear regression

intercept was shifted to 66 °C from that of 56 °C in the case of milk plus P_i (Table 1). The casein phosphate nanocluster content for milk plus P_i and Ca was also lower compared with skimmed milk at 25 °C. As the heating temperature increased, the difference in casein phosphate nanocluster contents between the milk and mineral supplemented samples was eliminated when they reached similar values ($P< 0.05$) at 60 °C (Fig. 1). This trend was also evident from the slopes of the two regression lines, i.e., at lower temperature (25–65 °C) milk plus P_i and Ca had the higher slope (0.0028 for milk plus P_i and Ca versus 0.0011 for skimmed milk) while at higher temperatures (70–80 °C) both slopes were virtually similar (0.0066 for milk plus P_i and Ca versus 0.0085 for skimmed milk). No significant difference in the NMR signal was observed while recovering at 25 °C following heat treatment (Fig. 4G) while in the FTIR spectrum (Fig. 4H) it was possible to observe an increase in the casein phosphate nanocluster signal.

4. Discussion

Previous studies have highlighted the effects of different heat treatments on milk minerals (de la Fuente, 1998). Most studies show that mineral equilibria shift towards the colloidal phase as temperature increases (Pouliot et al., 1989a) and pH of milk reduces (On-Nom et al., 2010). Furthermore, it has also been shown that once temperature reaches 90 °C alterations to mineral equilibria are no longer reversible (Pouliot et al., 1989b; Zhang & Aoki, 1996). This difference in reversibility behaviour points to a different structure of casein phosphate nanocluster as a result of its exposure to higher temperatures (Gaucheron, 2005). Using advanced analytical techniques now available (^{31}P NMR and FTIR) to investigate milk and dairy products (Boiani et al., 2017; Boiani et al., unpublished), the authors set out to confirm the findings of previous workers and to elucidate some new perspectives on the influence of temperature on casein phosphate nanocluster structure and mineral equilibria.

250 *4.1. Influence of temperature*

251

252 Using ^{31}P NMR, it was possible to directly observe for the first time the influence of heating
253 on the ^{31}P NMR peaks depicting casein phosphate nanoclusters, P_i distribution in milk and ionic
254 charge. Figs. 2 and 3 clearly show how an increase in temperature not only influenced the
255 repartitioning of minerals between colloidal and soluble phase (Gaucheron, 2005), but also had two
256 additional effects. At temperatures >70 °C a shift of the P_i signal was visible (Fig. 2); such as shift
257 is usually associated with an increase in pH (Gonzalez-Jordan, Thomar, Nicolai, & Dittmer, 2015).
258 However, in this case increasing the temperature caused a pH reduction (Fig. 1) in line with the
259 observation of On-Nom et al. (2010). Hence, it is concluded that raising the temperature increases
260 the negative charge of P_i, as already predicted by (de la Fuente 1998). On the other hand, the casein
261 phosphate nanocluster signal shifts to lower ppm (Fig. 3), an effect that is associated with an
262 increase in P:Ca. This indicates that increasing temperature leads to a change in casein phosphate
263 nanocluster structure, and in particular, an increase in its phosphorus content (Boiani et al., 2017).
264 The non-linear nature of the NMR data obtained during heating (Fig. 1) revealed two
265 distinct phases relating to changes in phosphorus – a slow rate of precipitation during the first phase
266 as represented by the smaller slope of the linear regression line, and a more rapid rate of
267 precipitation during the second phase (Fig. 1). In this study, the change between the two phases
268 occurs close to 70 °C (Fig. 1), while previous research shows that it occurred at 80–90 °C (de la
269 Fuente, 1998). The lower intercept temperature observed in this study may be due to the protracted
270 period (4 h) that the samples were subjected to in the NMR instrument due to the long data
271 acquisition time compared with a maximum of 30 min used in other studies (Zhang & Aoki, 1996).
272 This protracted duration (4 h) may have exaggerated the influence of temperature. Follow-up
273 studies are, therefore, required with a view to adapting the above observations to the more
274 conventional milk heating regimes using the Pouliot approach. It is likely that the outcome of such
275 studies would result in a phase change similar to that detected in this study. Interestingly, it was also

found that addition of a calcium chelator such as the phosphorus to milk reduces the impact of temperature change (Fig. 1). An increase in soluble casein in the case of milk plus P_i (Fig 5) suggests that the first phase destabilisation of the micellar conformation takes place within a narrower temperature range (25–56 °C). Addition of Ca countered this destabilisation effect thus confirming that the calcium chelating effect of phosphate (i.e., in milk plus P_i) was the main reason for the difference. It was also shown that the response to changes in the concentration of individual components involved in the mineral equilibria depends on heating status i.e., the low and high temperature phases.

In the milk concentrate samples, micellar casein had a dominant influence at all temperatures (Fig. 1) with the result that the slopes of the two regression lines were different to those of skimmed milk (Table 1). However, the micellar casein influence was not the same over the two temperature phases. The greater impact occurred in the first phase where the slope of milk concentrate line was three times higher than that of skimmed milk. In the second phase, a 2× increase in the value of the slope was in direct response to the 2× increase in concentration of micellar casein. The addition of P_i to skimmed milk did not alter its first phase slope (Fig. 1; Table 1), even when the phase change occurred at an earlier temperature. However, during the second phase precipitation was slowed by the presence of P_i, as deduced from the reduced slope of this sample (Fig. 1; Table 1). When Ca was added to P_i, as in the milk plus P_i and Ca sample, the first phase showed a higher slope compared with skimmed milk. Thus, the casein phosphate nanocluster concentration of milk plus P_i and Ca was reduced relative to P_i at 25 °C when compared with that of skimmed milk. However, this difference reduced as the regression line converged at 60 °C with skimmed milk, thus suggesting that the precipitation of P_i and Ca occurred within the first phase, but not during the second.

4.2. Reversibility and recovery study

Using both NMR and FTIR it was possible to investigate the influence of prolonged thermal exposure on mineral distribution. While NMR analysis did not demonstrate any difference within skimmed milk on recovery at 25 °C (Fig 4A), a reduction in casein phosphate nanocluster was found in the FTIR spectra of the same sample (Fig 4B). This is probably due to the limitations of NMR at the micellar casein concentration of milk. In fact, when micellar casein was increased in concentration, the NMR was able to distinguish a decreased casein phosphate nanocluster concentration at higher temperature (Fig 4C) in line with the result obtained using FTIR (Fig 4D), and as already established by previous work (de la Fuente, 1998).

The apparent contradictory results obtained with NMR and FTIR in milk plus P_i may be explained by the increase in soluble casein found in this sample (Fig. 5C). In fact, NMR as a spectrophotometric technique is not intended to be used with colloidal systems such as milk (Belton & Lyster, 1991). Therefore, an increase in soluble casein would increase the signal of the casein on the spectra, without a real increase of the casein phosphate nanocluster concentration such that it is necessary to rely mainly on the result obtained from the FTIR in this instance (Fig. 4F). Hence, milk plus P_i and Ca is the only sample that showed an increase in the FTIR signal after temperature reversal to 25 °C (Fig 4H). This increase, however, was not detectable by NMR (Fig 4G), and it suggests that part of the precipitate of P_i and Ca is not generating new casein phosphate nanocluster, but forming inorganic calcium phosphate outside of the micellar casein that is only detectable by FTIR (Boiani et al., unpublished).

5. Conclusion

These results show how milk mineral equilibria are influenced in different ways by manipulation of its components in conjunction with thermal treatment. It is possible to speculate that during the first heating phase (25–60 °C) P_i and Ca precipitate within micellar casein to form new casein phosphate nanocluster or as inorganic calcium phosphate salt in the case of low micellar

casein concentration. During the second heating phase (60–80 °C), it would appear that changes to the micellar casein-casein phosphate nanocluster interaction predominates as a result of the increased negative charge of P_i. The authors believe that this is the first time that the influence of singular ionic constituents on milk mineral equilibria during heat treatment was made possible using advanced analytical techniques such as ^{31}P NMR and FTIR. This paper added useful knowledge regarding the interaction between the mineral-containing serum (P_i and Ca) phase of milk and the colloidal (micellar casein and casein phosphate nanocluster) phase.

Acknowledgement

The authors would like to acknowledge funding support under the Irish Department of Agriculture Food Institution Research Measure (FIRM) for this study. Mr. Mattia Boiani was the recipient of a Walsh Fellowship funding award by Teagasc.

References

Belton, P. S., & Lyster, R. L. J. (1991). ^{31}P nuclear magnetic resonance spectra of milk from various species. *Journal of Dairy Research, 58*, 443–451.

Boiani, M., McLoughlin, P., Auty, M. A., FitzGerald, R. J., & Kelly, P. M. (2017). Effects of depleting ionic strength on ^{31}P nuclear magnetic resonance spectra of micellar casein during membrane separation and diafiltration of skim milk. *Journal of Dairy Science, 100*, 6949–6961.

Corredig, M., & Dalgleish, D. G. (1996). Effect of temperature and pH on the interactions of whey proteins with casein micelles in skim milk. *Food Research International, 29*, 49–55.

Dalgleish, D. G., & Corredig, M. (2012). The structure of the casein micelle of milk and its changes during processing. *Annual Review of Food Science and Technology, 3*, 449–467.

de la Fuente, M. A. (1998). Changes in the mineral balance of milk submitted to technological treatments. *Trends in Food Science and Technology, 9*, 281–288.

Donato, L., & Guyomarc'h, F. (2009). Formation and properties of the whey protein/κ-casein complexes in heated skim milk – A review. *Dairy Science and Technology, 89,* 3–29.

Fox, P., Uniacke-Lowe, T., McSweeney, P., & O'Mahony, J. (2015). Heat-induced changes in milk. In P. Fox, T. Uniacke-Lowe, P. McSweeney, & J. O'Mahony (Eds.), *Dairy chemistry and biochemistry* (pp. 345–375). Cham, Switzerland: Springer.

Gaucher, I., Piot, M., Beaucher, E., & Gaucheron, F. (2007). Physico-chemical characterization of phosphate-added skim milk. *International Dairy Journal, 17*, 1375–1383.

Gaucheron, F. (2005). The minerals of milk. *Reproduction Nutrition Development, 45*, 473–483.

Gonzalez-Jordan, A., Thomar, P., Nicolai, T., & Dittmer, J. (2015). The effect of pH on the structure and phosphate mobility of casein micelles in aqueous solution. *Food Hydrocolloids, 51*, 88–94.

Holt, C. (2004). An equilibrium thermodynamic model of the sequestration of calcium phosphate by casein micelles and its application to the calculation of the partition of salts in milk. *European Biophysics Journal, 33*, 421–434.

Le Graet, Y., & Brule, G. (1982). Effect of concentration and drying on mineral equilibria of skimmilk and retentates. *Lait, 62*, 113–125.

Liu, D. Z., Dunstan, D. E., & Martin, G. J. O. (2012). Evaporative concentration of skimmed milk: Effect on casein micelle hydration, composition, and size. *Food Chemistry, 134*, 1446–1452.

McMahon, D. J., Brown, R. J., Richardson, G. H., & Ernstrom, C. A. (1984). Effects of calcium, phosphate, and bulk culture media on milk coagulation properties. *Journal of Dairy Science, 67*, 930–938.

On-Nom, N., Grandison, A. S., & Lewis, M. J. (2010). Measurement of ionic calcium, pH, and soluble divalent cations in milk at high temperature. *Journal of Dairy Science, 93*, 515–523.

379 Pouliot, Y., Boulet, M., & Paquin, P. (1989a). Observations on the heat-induced salt balance
380 changes in milk I. Effect of heating time between 4 and 90 °C. *Journal of Dairy Research, 56*, 185–192.
381
382 Pouliot, Y., Boulet, M., & Paquin, P. (1989b). Observations on the heat-induced salt balance
383 changes in milk II. Reversibility on cooling. *Journal of Dairy Research, 56*, 193–199.
384 Wahlgren, N. M., Dejmek, P., & Drakenberg, T. (1990). A ^{43}Ca and ^{31}P NMR study of the calcium
385 and phosphate equilibria in heated milk solutions. *Journal of Dairy Research, 57*, 355–364.
386 Zhang, Z. P., & Aoki, T. (1996). Behaviour of calcium and phosphate in bovine casein micelles.
387 *International Dairy Journal, 6*, 769–780.

Figure legends

Fig. 1. Changes of casein phosphate nanocluster ^{31}P NMR integral with changing temperature: ●, skimmed milk samples; ◆, skimmed milk samples concentrated 2× by microfiltration; ▲, skimmed milk samples with 10 mM orthophosphate; ◇, skimmed milk samples plus 10 mM orthophosphate and 10 mM calcium. The dotted lines represent the linear regression for the different phases; values of slope, R^2, the intercept of the regression line and the pH value of the sample after heating at 80 °C for 4 h are given in Table 1.

Fig. 2. Soluble phosphate ^{31}P NMR spectra obtained from skimmed milk at (from bottom to top) 25, 40, 60, 65, 70, 75 and 80 °C.

Fig. 3. Casein phosphate nanocluster ^{31}P NMR spectra obtained from skimmed milk at (bottom to top) 25, 60, 65, and 80 °C.

Fig. 4. Reversibility and recovery study using casein phosphate nanocluster ^{31}P NMR integral (A, C, E, G) and Fourier transform infrared spectra (B, D, F, H); the black lines show the results obtained at the different temperatures, the grey line shows the reversibility signal at 25 °C. Panels A and B, skimmed milk samples; panels C and D, skimmed milk samples that were 2× concentrated using microfiltration; panels E and F, skimmed milk samples plus 10 mM orthophosphate; panels G and H, skimmed milk samples with 10 mM orthophosphate and 10 mM calcium.

Fig. 5. Reducing sodium docecylsulphate polyacrylamide gel electrophoresis of: A, skimmed milk; B, skimmed milk 2× concentrated using microfiltration; C, skimmed milk plus 10 mM

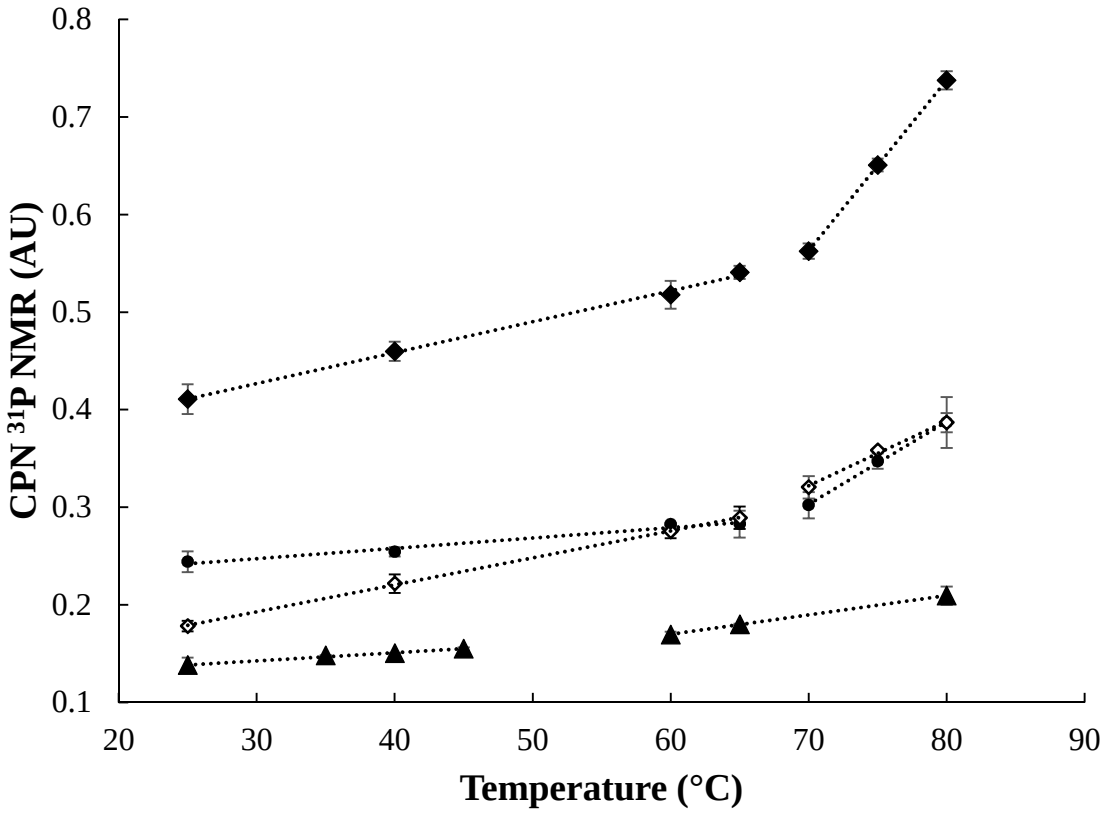
orthophosphate; D, skimmed milk plus 10 mM orthophosphate and 10 mM calcium. For each gel: lane 1, protein markers; lane 2, α_S-casein; lane 3, β casein; lane 4, skimmed milk; lane 5, sample; lane 6, supernatant of the sample obtained by centrifugation; lane 7, sample after heating at 80 °C for 4 h; lane 8, supernatant after heating at 80 °C for 4 h.

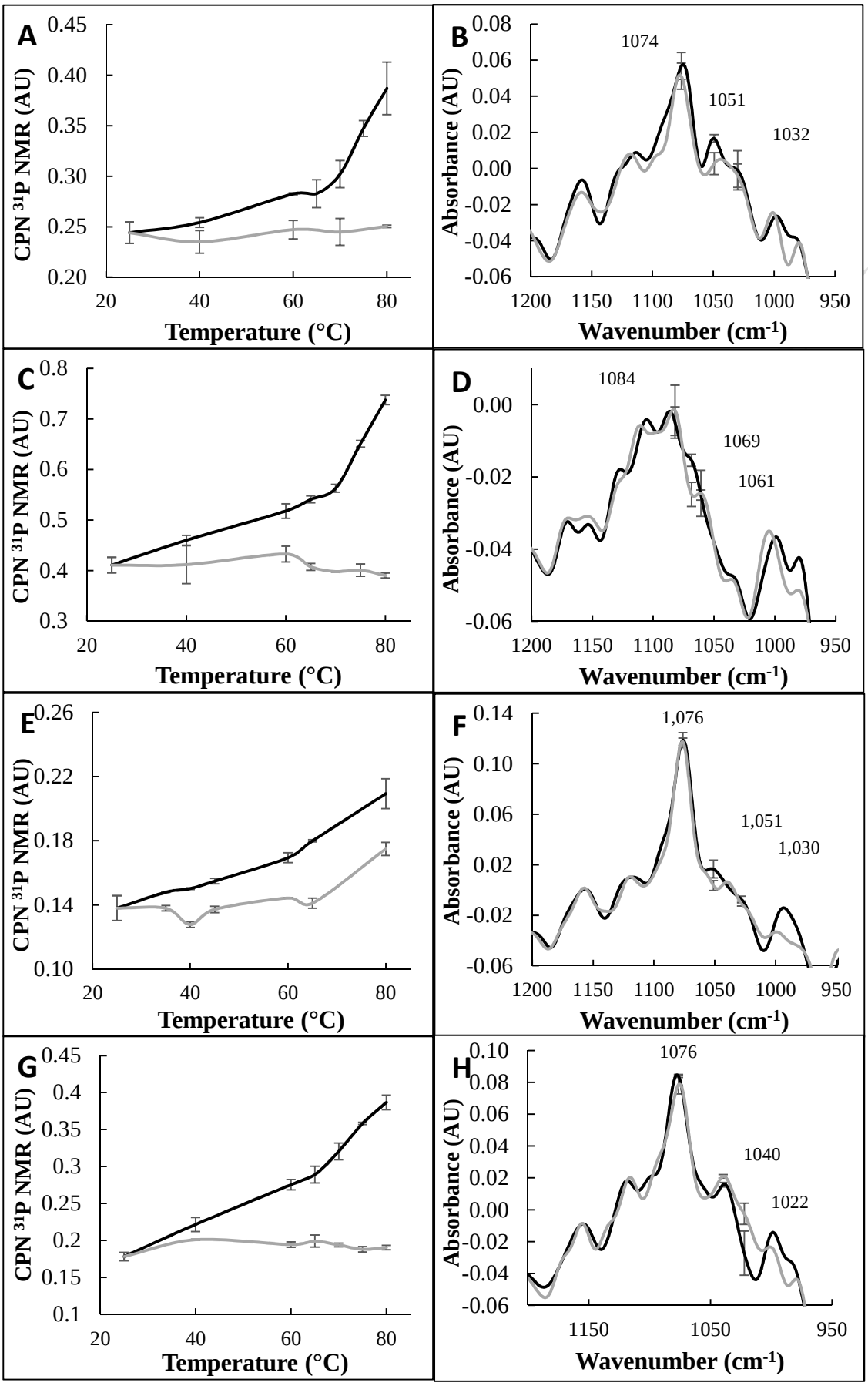
Table 1

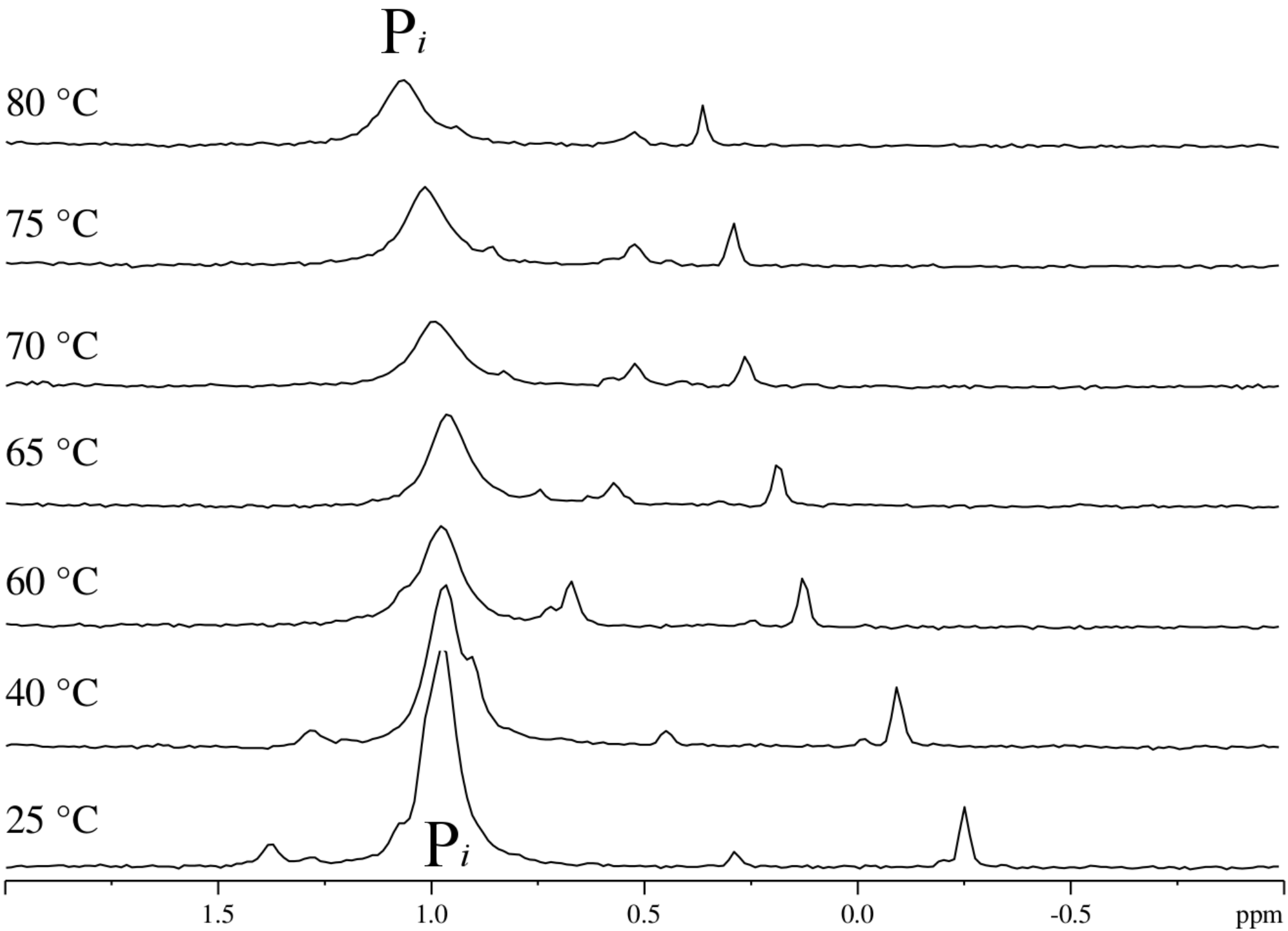
Changes of casein phosphate nanocluster ^{31}P NMR integral with changing temperature: values for slope, R^2, the intercept of the regression line and the pH value of the sample after heating at 80 °C for 4 h. [a]

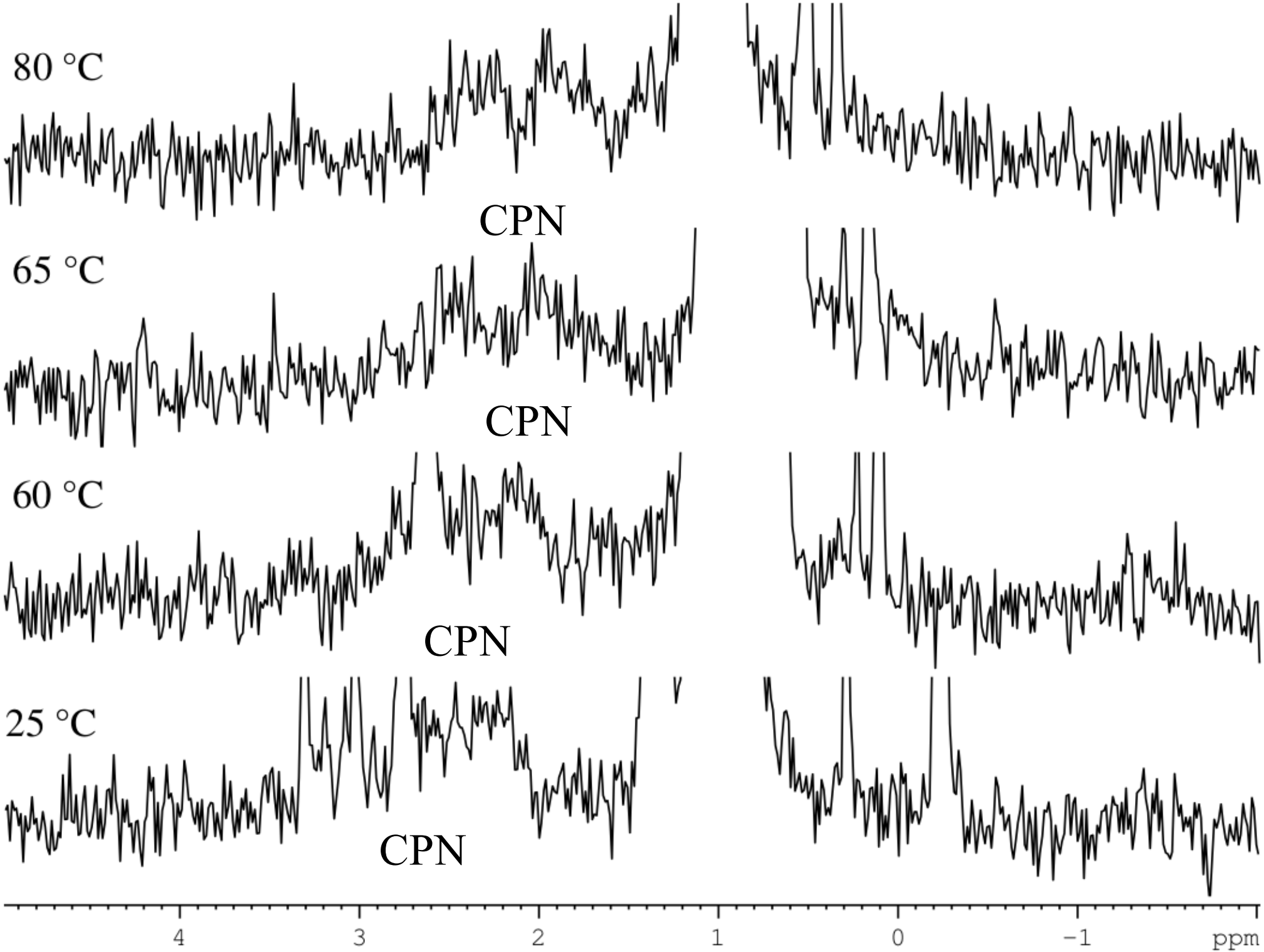
Parameter	M	M×2	M+P	M+P+Ca
Low T slope (CPN area $°C^{-1}$)	0.0011	0.0032	0.0008	0.0028
Low T regression line R^2	0.9723	0.9973	0.9859	0.9997
High T slope (CPN area $°C^{-1}$)	0.0085	0.0175	0.002	0.0066
High T regression line R^2	0.9986	0.9999	0.9998	0.9935
Intercept (°C)	68	69	56	66
pH	6.42 ± 0.03	6.47 ± 0.03	6.42 ± 0.01	6.34 ± 0.04

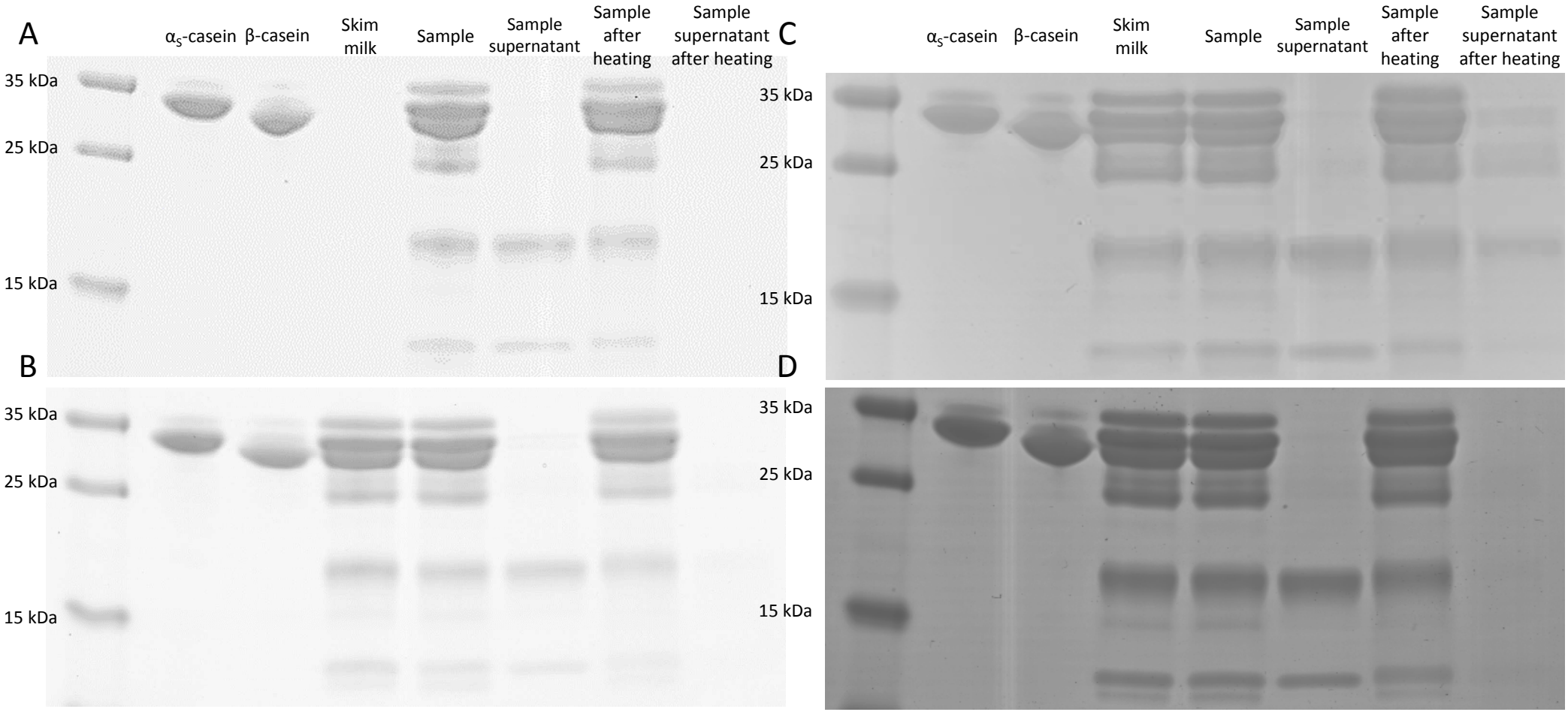
[a] Values were calculated from the data presented in Fig. 1. Abbreviations are: M, skimmed milk samples; M×2, skimmed milk samples concentrated 2× by microfiltration; M+P, skimmed milk samples with 10 mM orthophosphate; M+P+Ca, skimmed milk samples plus 10 mM orthophosphate and 10 mM calcium.




A
CPN 31P NMR (AU)
Temperature (°C)
B
Absorbance (AU)
1074
1051
1032
Wavenumber (cm-1)
C
CPN 31P NMR (AU)
Temperature (°C)
D
Absorbance (AU)
1084
1069
1061
Wavenumber (cm-1)
E
CPN 31P NMR (AU)
Temperature (°C)
F
Absorbance (AU)
1,076
1,051
1,030
Wavenumber (cm-1)
G
CPN 31P NMR (AU)
Temperature (°C)
H
Absorbance (AU)
1076
1040
1022
Wavenumber (cm-1)


Pi
80 °C
75 °C
70 °C
65 °C
60 °C
40 °C
25 °C
Pi
1.5
1.0
0.5
0.0
-0.5
ppm


80 °C
CPN
65 °C
CPN
60 °C
CPN
25 °C
CPN
4
3
2
1
0
−1
ppm


A
α_S-casein
β-casein
Skim milk
Sample
Sample supernatant
Sample after heating
Sample supernatant after heating
35 kDa
25 kDa
15 kDa
B
35 kDa
25 kDa
15 kDa
C
α_S-casein
β-casein
Skim milk
Sample
Sample supernatant
Sample after heating
Sample supernatant after heating
35 kDa
25 kDa
15 kDa
D
35 kDa
25 kDa
15 kDa